$^1$H NMR STUDY OF HETEROASSOCIATION OF CAFFEINE WITH ACRIDINE ORANGE IN AQUEOUS SOLUTION


The molecular mechanism of the action of caffeine (CAF) as a complexing intercepted of aromatic ligands intercalated in DNA is considered using a typical intercalant — acridine orange (AO) dye. Heteroassociation of CAF and AO was investigated by one- and two-dimensional $^1$H NMR spectroscopy (500 MHz). The concentration (at 298 and 308 K) and temperature dependences of the proton chemical shifts of molecules in aqueous solution were measured. The equilibrium constants of the CAF–AO heteroassociation reactions at different temperatures and the limiting chemical shifts of the protons of the aromatic ligands of the associates were determined. The most plausible structure of the 1:1 CAF–AO heterocomplex in aqueous solution is suggested based on the calculated values of the induced proton chemical shifts of the molecules and the quantum mechanical screening curves for CAF and AO. The thermodynamic parameters of CAF–AO heterocomplex formation are calculated. The structural and thermodynamic analyses indicate that dispersion forces and hydrophobic interactions play a significant role in heterocomplex formation in aqueous salt solution. The relative contents of different types of associate in a mixed solution containing CAF and AO are estimated. The equilibrium of CAF–AO heteroassociates in solution is characterized in relation to temperature. Heteroassociation of CAF and AO molecules leads to decreased effective concentration of intercalant in solution and hence to decreased mutagenic activity of the dye.

Caffeine (1,3,7-trimethylxanthine) is a psychomotor stimulant [1, 2]. It displays various cell effects; in particular, it inhibits reparation in bacterial systems [3, 4]. It is commonly recognized that the biological activity of caffeine is due to its interaction with biopolymers — proteins and DNA [4-6].

It was found [7-10] that chemoresistance of normal and cancer cells as well as their resistance to UV and X-radiation change differently under the action of caffeine. It was shown [11] that caffeine selectively alters the activity of the antibiotic adriamycin in cancer and normal tissues; in cancer cells, the effective concentration of the antitumor antibiotic in the presence of caffeine increases approximately twofold compared to the cells without caffeine. At the same time, experimental data show that caffeine inhibits the cytotoxic activity of ethidium bromide [12] and of a number of antitumor drugs such as doxorubicin and its analogs, ellipticine, etc. [13-17]. It was concluded [16-18] that caffeine forms complexes with aromatic molecules, decreasing their effective concentration and hence pharmacological activity. Thus it is maintained that caffeine acts as a complexing interceptor of aromatic biologically active substances binding to DNA by intercalation [17].

To verify this conclusion, heteroassociation of caffeine with aromatic molecules in solution was investigated and various models were suggested to interpret experimental data [17-22]. However, most of these models are inapplicable in the general case since they neglect formation of multidimensional aggregates of any size for both self-associates and heteroassociates in aqueous solution. Weller et al. developed a statistical thermodynamic model of association of two different aromatic substances forming infinitely dimensional aggregates in solution during both self-association and...
heteroassociation of molecules [23]. This model is difficult to use in practical applications because of the lack of analytical expressions convenient for analysis of experimental data. Recently, we developed a rather strict generalized model [24] taking into account formation of various n-dimensional aggregates in molecular self- and heteroassociation and using analytical expressions for NMR data for interacting molecules in mixed solution. This procedure enables one to determine the structural and thermodynamic parameters of heteroassociates from the experimental dependences of the proton chemical shifts of molecules on the concentration and temperature of solution.

Here the model is used to determine the structural and thermodynamic parameters of caffeine (CAF) heteroassociation with the aromatic dye of the acridine series — acridine orange (AO) in a 0.1 M phosphate buffer (pD 7.1) in order to examine the molecular mechanism of caffeine action as an interceptor of aromatic mutagenic substances. Previously, we investigated self-association of AO [25, 26] and caffeine [27] by NMR spectroscopy in analogous experimental conditions. Heteroassociation of CAF with AO was studied [17, 18] by optical spectroscopy. However, rather rough models were employed; the model suggested in [17] considered only the formation of a 1:1 heterocomplex but neglected self-association of aromatic molecules in solution, and the theoretical model of [18] neglected formation of various n-dimensional associates for all components in solution.

**EXPERIMENTAL**

Caffeine and acridine orange (Fig. 1) ("Sigma") were dissolved in D$_2$O (isotopic purity 99.95% D; "Sigma") and lyophilized. Solutions were prepared by adding the weighted sample in the 0.1 M deuterated phosphate buffer (pD 7.1) containing $10^{-4}$ mole/l EDTA. The concentration of aromatic molecules in aqueous solution was determined spectrophotometrically; the extinction coefficient $\varepsilon = 9740$ M$^{-1}$ cm$^{-1}$ ($\lambda = 273$ nm) for caffeine [28] and $\varepsilon = 5.6 \cdot 10^4$ M$^{-1}$ cm$^{-1}$ for AO ($\lambda = 492$ nm) [29].

1D and 2D $^1$H NMR spectra were measured on a Bruker DRX spectrometer with resonance frequency 500 MHz. The residual HOD signal was saturated during the detection period. The concentration measurements of the proton chemical shifts of molecules were performed at two temperatures (298 and 308 K); the temperature dependences of the proton chemical shifts of the aromatic ligands were measured in the temperature range from 278 to 353 K. The chemical shift was measured relative to DSS (2,2-dimethyl-2-silapentane-5-sulfoacid); tetramethylammonium bromide (TMA) was used as internal standard.

$^1$H NMR signal assignments and chemical and spatial bond identifications were accomplished using two-dimensional homonuclear TOCSY, NOESY, and ROESY experiments, respectively. Sample preparation and the experimental procedure are described in [26, 27].

**RESULTS AND DISCUSSION**

The structural and thermodynamic parameters of CAF–AO heteroassociation were determined by analyzing the concentration and temperature dependences of the chemical shifts of the nonexchangeable protons of both aromatic compounds in mixed solutions. The 2D-NOESY and 2D-ROESY spectra of mixed solutions did not display any